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<p>13. ABSTRACT (Maximum 200)</p> <p>Matrix metalloproteinase-2 (MMP-2) has been implicated in the progression of tumor invasion and metastasis. We have previously demonstrated that, in contrast with non-invasive cell lines, invasive breast cancer cells can activate exogenous MMP-2 when cultivated on collagen I gels. Although the $\alpha 2\beta 1$ integrin heterodimer is the principle cellular receptor for collagen type I, we were unable to block the collagen-induced activation of MMP-2 with antibodies against these integrin subunits. However, our experiments identified an exogenous serum factor whose presence was necessary for MMP-2 activation. We demonstrated a correlation between the invasive phenotype and the expression of osteonectin/secrated protein, acidic and rich in cysteine (SPARC). For invasive, but not non-invasive cells, recombinant SPARC (rSPARC) was able to induce MMP-2 activation in serum free medium and this activation was potentiated by exposure to collagen type I. We identified the peptide domain in SPARC responsible for this activity as localized to the N-terminal domain of the protein. Since rSPARC did not effect expression levels or post-translational modifications of MT1-MMP, the mechanism by which it activates MMP-2 remains to be elucidated.</p>			
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Introduction

Tumor invasion and metastasis formation involves loss of basement membrane integrity, and matrix-metalloproteinase-2 (MMP-2) has been implicated in this process. MMP-2 is secreted as a latent zymogen and requires the proteolytic removal of a profragment for its activation. MMP-2 activation appears to be mediated by membrane-type-MMP-1 (MT1-MMP), a transmembrane matrix metalloproteinase selectively expressed by invasive human breast cancer (HBC) cell lines. Although this subset of breast cancer cells express a basal level of MT1-MMP, they need additional signals to induce MMP-2 activation. Previous work has shown that three-dimensional gels of fibrillar type I collagen can stimulate MT1-MMP expression, and induce MMP-2-activation in these responsive HBC cell lines. Furthermore, we have shown by *in situ* hybridization on cancer specimens a co-localization of MT1-MMP and collagen type I mRNAs in fibroblasts close to invasive breast carcinomas, but not in benign breast tumors or normal tissue. A small number of fibroblasts at specific foci around pre-invasive lesions also selectively co-express type I collagen and MT1-MMP, although most are negative for both. Such a focal localization strongly support the *in vivo* relevance of the collagen induction pathway, and suggest that it may contribute to breast tumor progression. **Our hypothesis is that type I collagen induction of MT1-MMP represents a major MMP-2-activation inducing pathway *in vivo*, and that interference with this mechanism may inhibit the growth and/or metastasis of breast cancers.** We have therefore proposed to (i) manipulate collagen levels around HBC cells in culture models or xenografted in nude mice, either by direct co-inoculation of collagen or by treatment with anti-fibrotic agents, and examine the MMP-2-activation, growth, invasion and metastasis of these cells, (ii) identify cell surface receptors implicated in the transduction of the collagen response leading to MMP-2 activation and MT1-MMP overexpression, and (iii) perform PCR differential display on a large panel of responsive versus non-responsive breast cancer cell lines to elucidate additional genes which are regulated by collagen and may contribute to the collagen-induced MT1-MMP stimulation, MMP-2-activation, or overall invasive phenotype.

Methods

Materials

Recombinant human SPARC (rSPARC) was produced in *E. coli* and purified as previously described (Bassuk *et al.*, 1996). The synthetic peptides used in this study are a series of 20-21 mers spanning different regions of the mouse or the bovine SPARC amino acid sequence and were synthetized and purified by HPLC (Lane and Sage, 1990)

Cell culture

The six human mammary cancer cell lines used in this study were obtained originally from the ATCC (Rockville, MD, U.S.A.). Four cell lines (MDA-MB-435, MDA-MB-231, BT549 and Hs578T) display high *in vitro* invasive abilities and have lost some epithelial features, such as E-cadherin expression. In contrast, two cell lines (MCF-7 and T47D) have retained many epithelial features and do not display highly invasive properties. The MT1-MMP-transfected cell lines (#2-6 and #8-5) were isolated after transfection of MCF-7 cells with a human MT1-MMP cDNA in the CMV-promotor driven, neomycin-resistance vector pCNCXh. The MT1-MMP transfected clones partially activate MMP-2 to the 62kDa intermediate in a concentration dependent manner, and this intermediate is activated further to the 59kDa mature form in the presence of ConA (Pulyaeva *et al.*, in preparation). All cells were cultivated in IMEM supplemented with 10 % fetal bovine serum (FBS). Cultures were maintained at 37°C. All chemicals and culture media were purchased from Sigma (St-Louis, MO, USA) or Gibco (Gaithersburg, MD, USA).

Blocking activation of MMP-2 using anti-integrin antibodies

Blocking experiments were performed using the following anti integrins antibodies: anti- $\alpha 2$ integrin antibody clone P1E6 (Chemicon, Temecula, CA), anti- $\alpha 2$ integrin antibody clone 6F1 (provided by Dr. Barry S. Coller, Mount Sinai Medical Center, N.Y.), anti- $\beta 1$ blocking antibody clone P5D2 (Chemicon, Temecula, CA), and anti- $\beta 1$ blocking antibodies clone Mab13 (provided by Dr. Steven Akiyama, NIDR, NIH). Blocking experiments were performed in 24-well plates. Cells (5×10^4) were preincubated with the antibodies (at concentrations ranging from 1 to 10mg/ml for P5D2 and P1E6, and up to 50 μ g/ml for Mab13) for 30min at room temperature in IMEM containing 5% FBS. They were plated either on plastic in 10% FBS-IMEM with the anti-integrins antibodies for 24 hours or were

placed inside a collagen type I solution (250 μ l per well at 2mg/ml collagen: Vitrogen, Palo Alto, CA, U.S.A.) also containing the anti-integrins antibodies. After polymerization of the collagen, the gels were submerged for 24 h in IMEM containing 10% FBS with the same concentration of antibodies as that within the gels. The cells plated either on plastic or in collagen gels were then washed twice in serum-free medium (SFM) (IMEM supplemented with 1% HEPES buffer, 1% trace elements, 1% non-essential amino acids, and 0.4% Insulin-Transferin-Selenium mixture) and were incubated for 72 h in a SFM/MMP-2 mixture containing SFM (75%) and SFM conditioned for 72 h by MMP-2-transfected MCF-7 cells (25%). The antibodies were again added to the SFM/MMP-2 medium.

rSPARC and SPARC peptide stimulation-preparation of conditioned media

Induction experiments with rSPARC or with SPARC peptides were performed in 48-well plates. For stimulation on plastic, 3×10^4 cells were plated in 10 % FBS containing IMEM for 4 h at 37°C to allow adhesion. The cells were then washed twice in SFM and were incubated for 72 h in the SFM/MMP-2 mixture. Different concentrations of rSPARC or SPARC peptides were added to the SFM/MMP-2 medium. For stimulation in collagen gels, the cells were mixed into a collagen type I solution (at 2mg/ml: Vitrogen) before 150 μ l of the mixture was placed into each well (at a concentration of 3×10^4 - 5×10^4 cells/ 150 μ l). rSPARC or the different peptides were added to the into the gel before polymerization at 37°C for 30 min. After polymerization, 150 μ l of the SFM/MMP-2 mixture was added on top of the gels with the same concentration of rSPARC or peptides as that within the gel. After 72 hours, the conditioned media were collected for zymography and/or TIMP-2 ELISA analysis (see below). The cells were also incubated with fresh SFM containing the Wst-1 proliferation reagent (Boehringer Manheim, used as recommended by the manufactor) for 1h at 37°C. The medium was transferred into a 96 well plate, and the optical density was read at a wavelength of 450 nm.

Gelatin zymography assays

Samples were denatured under non-reducing conditions and were resolved on 10% sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS-PAGE) gels containing 0.1 % (weight/volume: w/v) gelatin. The gels were washed 1 h at room temperature in a 2 % (volume/volume: v/v) Triton X-100 solution, transferred to a buffer containing 10 mM CaCl₂,

50 mM Tris-HCl, pH 7.6 and incubated overnight at 37°C. The gels were then stained for 30 min with 0.1 % (w/v) Coomassie blue (G 250) in 45 % (v/v) methanol / 10 % (v/v) acetic acid and were de-stained in 10 % (v/v) acetic acid / 20 % (v/v) methanol.

Northern blot analysis

For Northern blotting, experiments with the different SPARC peptides were scaled up into 6-well plates (30×10^4 - 50×10^4 cells were used and placed in a volume of 1.5 ml) and were performed as described above. After 72h, the conditioned media were collected for zymogram and/or for TIMP-2 analyses, and the cells were collected for Northern blotting. The cells were recovered from the gels by treatment with collagenase (type 1A, 1 mg/ml in PBS: Sigma, St-Louis, MO, U.S.A.). For analysis SPARC mRNA expression by the breast cell lines, total RNAs were isolated from subconfluent cells cultivated in standard culture conditions. Total RNAs was extracted with guanidium isothiocyanate and purified on a CsCl cushion (Chirgwin *et al.*, 1979). Four μ g of each RNA was analyzed by electrophoresis on 1 % agarose gels containing 10 % formaldehyde and was subsequently transferred onto nylon membranes (Hybond TM-N, Amersham, Aylesbury, UK). The membranes were hybridized with a cDNA probe for either MT1-MMP (provided by Dr. Motoharu Seiki, Kanazawa University, Japan), TIMP-2 (provided by Dr. William G. Stetler-Stevenson, NCI, NIH) or SPARC (provided by Dr. Larry Fisher, NIDR, NIH), labeled with 32 P by a random-priming labeling kit (Boehringer Mannheim, Germany). The amounts of blotted RNA were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH: Clontech, Palo Alto, CA, U.S.A.). The hybridization signals were quantified by Phosphorimager 441 SI (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Western blotting analyses

Analyses of SPARC expression in the breast cancer cell lines were performed on 72 h-conditioned media (10 ml in a 10 cm Petri dish) obtained from cells cultured on plastic in SFM. Loading was normalized with respect to cell density, estimated by cell counting after the 72 h-incubation.

For MT1-MMP analyses of cells stimulated with SPARC peptides (see above), the assays were performed in 24 well plates (15×10^4 - 25×10^4 cells in 300 μ l). After 72 h, conditioned media were collected for zymographic analyses and the cells were reserved for

protein extraction. Total protein lysates were prepared in a HEPES buffer [50mM HEPES pH 7.5, 150 mM NaCl, 10 % glycerol, 1 % Triton X 100, 1.5 mM MgCl₂, 1 mM EGTA (pH 8.0)] containing protease inhibitors (Merck, Darmstadt, Germany) (1 mM phenylmethanesulfonylfluorid (PMSF), 10 µg/ml leupeptin and 10 µg/ml aprotinin). Protein concentration was determined with the BCA kit (Pierce, Rockford, IL) and 25 µg were applied to the gel.

Samples were mixed with 1/5 sample buffer [0.31 M Tris (pH 6.8), 10 % SDS (w/v) 25 % glycerol (v/v), 12.5 % b-mercaptoethanol (v/v), 0.125 % bromophenol blue (w/v)], boiled for 5 min, separated on a 10 % SDS-PAGE gel, and transferred to a nitrocellulose filter (Hybond-ECL membrane, Amersham, Arlington Heights, IL). Transfer was monitored with Ponceau red reversible staining. The filters were blocked in 5 % milk in PBS for 4 h before exposure to the primary antibody overnight at 4°C to the rabbit polyclonal anti-SPARC G-protein sepharose purified IgG (1/500: provided by Dr. Larry Fisher NIDH, NIH) or mouse monoclonal anti-MT1-MMP antibody (10 µg/ml; clone 118 kindly provided by Dr. Motoharu Seiki, Tokyo, Japan). The membranes were then incubated with a secondary horseradish-peroxydase (HRP)-conjugated goat anti-rabbit or goat anti-mouse antibody (1/2500: Amersham, Arlington Heights, IL). Signals were developed using an enhanced chemiluminescence (ECL) kit (Amersham, Arlington Heights, IL).

TIMP-2 ELISA

Human recombinant TIMP-2 was produced in Chinese Hamster Ovary cells (CHO) transfected with pDSRa2 vector containing human TIMP-2 cDNA (provided by Dr. Yves DeClerck, Children Hospital, Los Angeles, CA). Recombinant TIMP-2 was purified further as described (DeClerck *et al.*, 1991) and was used to establish a standard curve for the ELISA and to generate polyclonal antibodies in both rabbit and chicken embryonated eggs. The chicken anti-TIMP-2 IgY were purified by ammonium sulfate precipitation. Rabbit polyclonal antibodies were purified by affinity column chromatography.

For ELISA test (Noël *et al.*, 1998), rabbit polyclonal anti-TIMP-2 antibodies were coated (dilution 1/750) in 96-well ELISA plates. Recombinant TIMP-2 (standard curve from 20ng/ml to 0.3ng/ml) or conditioned media samples were then incubated in the wells. Fixed TIMP-2 was detected in two steps with the chicken anti-TIMP-2 antibody, followed by an incubation with an HRP-conjugated rabbit anti-chicken antibody.

Results

We had previously shown that, in contrast with non-invasive cell lines, invasive breast cancer cells activate exogenous MMP-2 when cultivated on collagen type I gels. Cells were plated for 24 h with serum-supplemented IMEM. After this they were incubated with MMP-2 supplemented serum-free medium for 72 h. Our goal was to examine the mechanism through which these cells respond to the collagen gel and to determine the level at which the differential response occurs between non-invasive and invasive cells.

Anti-integrin antibodies are unable to block collagen-type I/serum-induced MMP-2 activation

Since $\alpha 2\beta 1$ is the principal cellular receptor for collagen type I, we tried to block collagen-induced activation of MMP-2 with anti- $\alpha 2\beta 1$ antibodies. We used two invasive breast cancer cell lines, MDA-MB-231, which did not demonstrate an increase in MT1-MMP mRNA level on collagen gels, and BT549 which showed the greatest increase of MT1-MMP mRNA on collagen gels, as previously described (Gilles *et al.*, 1997). Seltzer *et al.* (1994) had shown an inhibition by $\alpha 2\beta 1$ antibodies of MMP-2 activation induced in fibroblasts cultivated within collagen type I gels. Following their procedure, we cultivated our breast cells under the same conditions and used the same antibodies. These antibodies inhibited the adhesion of both cell lines on collagen type I. However, we were unable to block the activation of MMP-2 in either breast cancer cell line. Furthermore, we observed that a 24 h preincubation with serum was necessary to obtain activation. Indeed, cells plated directly in collagen gels in serum-free medium remained viable, but did not activate MMP-2 (data not shown). These data suggested the presence in serum of an exogenous factor(s) necessary for MMP-2 activation in these cells.

SPARC expression in human mammary cancer cell lines

We examined the possibility that SPARC is involved in the collagen-induced MMP-2 activation because (1) it is present in serum, (2) it has been shown to inhibit cell spreading through an RGD-independent pathway (3) it binds collagen type I, and (4) it has been shown to regulate expression of other MMP in fibroblasts and human monocytes (Tremble *et al.*, 1993; Shankavaram *et al.*, 1997). We examined the expression of SPARC in several human breast cancer cell lines displaying various degrees of invasion and MMP-2 activation. We

found that SPARC mRNA was prevalent in invasive BT549 and Hs578T cells, relatively diminished in invasive MDA-435 cells, and undetectable in non-invasive T47D or MCF7 cells or in invasive MDA-231 cells. Similar results were seen with respect to SPARC protein, except that SPARC protein could not be detected in the conditioned medium of the MDA-435 cell line, which showed a low level of mRNA.

Induction of MMP-2 activation by SPARC

We next looked at the ability of these two cell lines to activate exogenous MMP-2 in the presence of different concentrations of rSPARC in the absence of serum. rSPARC induced MMP-2 activation by MDA-MB-231 and BT549 cultured in collagen gels or on plastic in a concentration-dependent manner. Cultivation of the MDA-231 cells in collagen gels clearly potentiated the response to SPARC, since MMP-2 activation could be detected at the lowest concentration (25 µg/ml) in collagen gels, whereas the highest dose (100µg/ml) was necessary to induce MMP-2 activation on plastic. The effect of rSPARC on BT549 cells cultured on plastic appeared to be stronger than that seen for cells in collagen. In contrast to the results obtained with invasive breast cancer cell lines, we did not observe any MMP-2 activation in non invasive MCF-7 cells treated with rSPARC, whether they were cultured on plastic or in collagen gels.

Induction of MMP-2 activation by different SPARC peptides

To define the domain(s) in SPARC responsible for this activity, we monitored MMP-2 activation of MDA-231 and BT549 cells exposed to several synthetic peptides from the different domains of the SPARC molecule. We mainly used peptide 1.1 (N-terminal domain I), peptide 3.2 (domain III), and peptide 4.2 (C-terminal domain IV). Peptide 1.1 potentiated MMP-2 activation in both MDA-231 and BT549 cells on plastic or in collagen type I gels. In contrast, we did not see any enhancement with the other two peptides. As seen with rSPARC, the dose response to peptide 1.1 was better in both cell lines on collagen gels relative to plastic. Also, none of the three peptides induced MMP-2 activation in the non-invasive MCF7 cell line.

Peptide 1.1-induced MMP-2 activation: modulation of MT1-MMP mRNA level

Since MT1-MMP is considered a major MMP-2 activator, we investigated the effect of SPARC on MT1-MMP expression. Using Northern blotting, we examined the steady-state levels of MT1-MMP in MDA-231 and BT549 cells treated with peptide 1.1, in collagen gels or on plastic. As previously reported (Gilles *et al.*, 1997), MT1-MMP mRNA was expressed by both cell lines. We also observed increased levels of MT1-MMP mRNA in BT549 cells cultivated in collagen gels, however, exposure to peptide 1.1 did not increase the basal level of MT1-MMP in either cell line, whether it was cultured in collagen type I gels or on plastic.

Peptide 1.1-induced MMP-2 activation: modulation of MT1-MMP protein level

Since we could not detect significant changes in MT1-MMP mRNA by peptide 1.1 in either of the two cell lines, we examined potential post-translational modifications of MT1-MMP by Western blotting. Three major bands of 63, 60 and 43 kDa have been described for MT1-MMP, which are thought to represent the proenzyme and two forms resulting from its activation (Lohi *et al.*, 1996). We were unable to detect any clear difference on Western blots in MDA-231 cells treated with peptide 1.1 relative to cells treated with peptides that did not induce MMP-2 activation on plastic or in collagen gels. The 43 kDa band was detectable in all samples, although it was weak in the untreated cells, especially those on plastic. In BT549 cells, a clear increase of the 60 kDa and the 43 kDa forms was observed in the cells cultivated in the collagen gels relative to the cells cultured on plastic, in agreement with our results obtained by Northern blotting. However, as seen with MDA-231 cells, no qualitative changes in MT1-MMP could be associated with the induction of MMP-2 activation.

Induction of MMP-2 activation by peptide 1.1 in MCF7 cells transfected with MT1-MMP

We have recently introduced MT1-MMP into MCF7 cells that lack endogenous MT1-MMP (Pulyaeva *et al.*, manuscript in preparation). Since our Northern blotting results implied that MT1-MMP is not participating in the SPARC-induced MMP-2 activation, we examined MMP-2 activation and MT1-MMP expression in these MCF7 cells transfected with MT1-MMP. MT1-MMP transfected-clones already activated MMP-2 under basal conditions on plastic (no MMP-2 activation or MT1-MMP protein was detected in the vector control #3-1 cells). As observed for MDA-231 and BT549 cells, activation was enhanced in cells treated with the peptide 1.1, supporting the involvement of MT1-MMP in the mechanism. However, no qualitative or quantitative changes of MT1-MMP expression were identified by Western

blotting, in agreement with our data for MDA-231 and BT549 cells. These data differ from those in the literature that describe transcriptional or post-translational regulation of MT1-MMP associated with MMP-2 activation. Therefore, we verified by Western blotting that Concanavalin A, an established activator of MMP-2, had the reported effects. We confirmed previously published results that ConA induces an overexpression of the 63 and 60 kDa forms of MT1-MMP (Pulyaeva *et al.*, 1997; Yu *et al.*, 1995, 1997a,b). Furthermore, we also observed the appearance of the 43 kDa form associated with the induction of MMP-2 activation in all the cell lines including the MT1-MMP transfectants, as previously described in fibrosarcoma HT1080 cells (Lohi *et al.*, 1996).

rSPARC and peptide 1.1 induced a decrease in soluble TIMP-2

Since we could not find any apparent modifications in MT1-MMP associated with the SPARC/collagen induced-MMP-2 activation, we looked at levels of TIMP-2 in the conditioned media of the cells treated with rSPARC and SPARC peptides on plastic and in collagen gels. We demonstrated a clear concentration-dependent diminution of TIMP-2 levels in the samples treated with rSPARC, both on plastic or in collagen, concomitantly with increased MMP-2 activation. Similarly, a decreased level of soluble TIMP-2 was found in the samples treated with peptide 1.1. This decrease was not seen in the samples treated with peptide 3.2, one of the peptides that does not induce MMP-2 activation. The decreased levels of TIMP-2 protein were not correlated with decreases in TIMP-2 mRNA.

Both rSPARC and peptide 1.1 induced a diminution of cellular metabolism. This observation was confirmed in a proliferation assay, and was particularly true at high doses of rSPARC (100 μ g/ml) and rSPARC peptide (above 1mM). However at lower doses, this antiproliferative effect was not sufficient to be responsible for the decrease in TIMP-2 protein that we observed.

CONCLUSIONS

The results from aim 2 indicate that direct ligation with $\alpha 2\beta 1$ integrin does not seem to be mediating the collagen mediated MMP-2-activation in these cells, at least under the conditions examined. This was not in accordance with the predicted results. In our hands the antibodies examined inhibited cell spreading. We determined however, that there is a serum factor involved in MMP-2 activation and identified the matricellular protein SPARC in the collagen regulation of MMP-2-activation. Identification of the differential regulation of SPARC between cells of invasive and non-invasive phenotype satisfied the criteria set out in aim 3 and identifies a gene product that contributes to collagen-induced MMP-2 activation. The component mediated by SPARC appears to be the non-transcriptional component required in MT1-MMP mediated MMP-2 activation we have described previously but that until now had remained unidentified.

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